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Plasticity in the contribution of T cell receptor variable region residues to binding of peptide-HLA-A2 complexes

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Abstract

One hypothesis to account for MHC-restriction by T cell receptors (TCRs) holds that there are several evolutionary-conserved residues in TCR variable regions that contact MHC. While this 'germline-codon' hypothesis is supported by various lines of evidence, it has been difficult to test. The difficulty stems in part from the fact that TCRs exhibit low affinities for pep/MHC, thus limiting the range of binding energies that can be assigned to these key interactions using mutational analyses. To measure the magnitude of binding energies involved, here we used high-affinity TCRs engineered by mutagenesis of CDR3. The TCRs included a high-affinity, MART-1/HLA-A2-specific single-chain TCR and two other high-affinity TCRs that all contain the same Vα (HLA-A2), with different peptides and Vβ regions. Mutational analysis of residues in CDR1 and CDR2 of the three Vα2 regions showed the importance of the key 'germline codon" residue Y51. However, two other proposed key residues showed significant differences among the TCRs in their relative contributions to binding. Using single-position, yeast-display libraries in two of the key residues, MART-1/HLA-A2 selections also revealed strong preferences for wild-type 'germline codon' residues, but several alternative residues could also accommodate binding and hence, MHC-restriction. Thus, although a single residue (Y51) could account for a proportion of the energy associated with positive selection (i.e. MHC-restriction), there is significant plasticity in requirements for particular side-chains in CDR1 and CDR2 and in their relative binding contributions among different TCRs.

Keywords

T cell receptors; single-chain; yeast display; directed evolution; germline codon bias

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Introduction

T cell-mediated recognition of a foreign peptide bound to a product of the major histocompatibility complex (MHC) occurs through the αβ T-cell receptor (TCR), in a process that is referred to as MHC-restriction. TCRs contain six complementarity determining regions (CDR), three (CDR1, 2, and 3) in each α and β chain. CDR1 and 2 loops are "germline derived" since they reside in the region encoded by each variable region gene, which do not undergo either somatic mutation or rearrangements. CDR3 loops are more variable in sequence as they are encoded by the junctions of somatically rearranged gene segments (VJ in the α locus, and VDJ in the β locus), similar to antibodies.

In virtually all of the structures of TCR:pep/MHC complexes, the CDR1 and 2 loops are found to dock over the helices of the MHC protein, whereas the CDR3 loops are positioned over the peptide where they can contribute most importantly to the antigen specificity of the reaction[1; 2]. Despite these generalizations, there are cases where CDR1 residues are near the peptide and CDR3 residues appear to contact the MHC protein[3–6].

While there is conserved, diagonal docking of TCRs over pep/MHC ligands, the complexities associated with the diversity of TCRs, peptides, and MHC proteins have made it difficult to reveal conserved chemistries of the interactions that could account for the process of MHC restriction. More recent evidence, where the structures of TCRs with the same V regions and the same MHC restricting elements have been compared, has led to the "germline codon" hypothesis. In this hypothesis, several key residues in CDR1 and/or CDR2 interact invariably with residues from the MHC protein, suggesting that these TCR residues evolved to establish the biochemical basis of MHC-restriction. Results of mutagenesis of these residues have been consistent with their contribution to binding[7–11], or even in the process of positive selection[12]. However, these studies have been unable to reveal the energetic importance of the residues among different TCRs.

A recent study suggested that there is no absolute requirement for specific residues within the germline Vα or Vβ loops, as it was possible to modify these very significantly and still achieve MHC-restriction and positive selection in the thymus[13]. Whether these modified TCRs retained the diagonal footprint characteristic of conventional TCRs remains to be seen. Another study showed that the CDR3 regions can significantly alter the germline-encoded interactions[14].

Studies with the mouse 2C system showed that two of the predicted "germline codon" residues in CDR2β (Y46 and Y48) were unable to bind the SIY/K^b antigen when the residues were changed to alanines[7; 15]. More recent work with a high-affinity mutant of the mouse 2C (m33), generated in CDR3α, showed that the TCR docked in an identical way as the wild-type TCR, and that all of the CDR1 and CDR2 contacts were preserved[16; 17]. In addition, both the 2C TCR and the m33 TCR had the same amino acid preferences at Y46, using a T-cell library based approach[18]. To the best of our knowledge, there have not been studies with human TCR single-site mutants to understand the issue of CDR1 and CDR2 contributions to binding properties, in large part because wild-type affinities are already so low. This prompted us to more fully exploit the idea of using high-affinity TCRs to determine the requirements and binding energies associated with MHC restriction in a human system.

Recently, we described the engineering of two human single-chain TCRs, 868 and A6, with high-affinity for HLA-A2-restricted peptides HIV (SL9 or Gag) and Tax, respectively[19]. This study showed that the Vα2 (IMGT:TRAV12) region was stable compared to other V regions, and that a polymorphism in framework residue 49 of Vα2 facilitated the display, engineering, and expression of soluble scTv fragments in *E. coli*. Using this as a platform

for work with additional human TCRs, here we have engineered a third Vα2-containing scTv against the MART- 1/Melan-A peptide restricted by HLA-A2; MART-1 is a human differentiation antigen expressed in more than 80% of metastatic melanoma tumors[20]. Collectively, these three TCRs, with the same CDR1α and CDR2α, provided an opportunity to examine the energetic impact of several key residues. In addition, there are several published crystal structures of Vα2-containing TCR:pep-HLA-A2 complexes[3–5; 21], including the A6 TCR, providing a structural framework for the interpretation of the results.

Using a combination of alanine-scanning mutagenesis, and library-based selections with pep/HLA-A2, our findings indicated that: 1) two of the proposed CDR1 germline codon residues, R28 and Q31, contributed to binding in some cases, but not in others, consistent with recent structural studies suggesting that CDR3 editing can influence CDR1 contacts[14], 2) one of the germline codon residues, Y51, exhibited similar and significant contributions to binding in all three cases (~1.5–2 Kcal/mol in free energy); the free energy contributions of this residue, together with that contributed by one of the CDR1 residues, would be sufficient to produce an equilibrium binding constant (K_D value ~ 1 mM; ΔG ~ 4 Kcal/mol) associated with co-receptor dependent positive selection; and 3) using a library approach, the tyrosine at position 51 was preferred for pep-HLA-A2 binding, relative to almost all other amino acids, although several conserved side chains (Phe, Trp) could be substituted. Thus, there is plasticity in the binding requirements of the germline encoded residues, but the evidence suggest that there has been evolutionary pressure to maintain certain key residues.

Results

Affinity-engineering a human TCR against MART-1/HLA-A2

The Vα2-positive MART-1-reactive T cell clone, INRi-T1 (referred to as T1), was isolated from a melanoma-reactive T-cell line. In order to engineer a receptor with improved binding affinity, the V regions were cloned as a single-chain TCR fragment (scTv) in the orientation Vβ-linker-Vα into a yeast-display vector. The scTv was expressed on the surface of yeast via an N-terminal Aga2 fusion followed by a hemagglutinin (HA) epitope tag for detection of the displayed protein (Fig. 1A). Additionally, a F49S mutation was introduced into the Vα2-domain of the construct to confer thermal stability to the scTv, as described previously[19].

Induced yeast cells containing the T1 scTv fusion (template) were positive with an anti- HA epitope antibody (Fig. 1B). In order to detect individual Vα and Vβ domains, anti-Vα2 and anti-Vβ16 monoclonal antibodies were used for staining. Positive staining was seen with the anti-Vα2 antibody and weak staining was observed with the anti-Vβ16 antibody. Temperature stability assays have suggested that the Vβ16 antibody recognizes a conformational epitope whereas the Vα2 antibody recognizes a linear epitope (data not shown). This suggested that most of the T1 scTv fragments were not properly folded on the surface of yeast.

To generate a more stable T1 scTv that would be expressed on the surface of yeast, we performed random mutagenesis and selected for clones with improved surface expression by binding to the anti-Vβ16 antibody. Previous work with mouse TCRs 2C and 3L.2 and human TCRs A6 and 868 have shown that selection of scTvs with enhanced yeast surface expression and temperature stability also correlate with increased soluble expression[19; 22; 23]. Following two rounds of sorting, several mutants that had improved binding to the anti-Vβ16 antibody were isolated. Clones were screened for resistance to thermal denaturation using the Vβ16 as a probe (data not shown), and the scTv mutant T1-S18 was selected for use as a template for affinity engineering (Fig. 1B, middle panels). The

T1-S18 scTv was sequenced and shown to contain four mutations: two in the Vα (S40P and Q80R), one in the Vβ (V80I), and one in the linker (K8E)(Supplementary Fig. 1).

Engineering of a high affinity surface displayed T1 scTv fragment via site directed mutagenesis

Degenerate libraries were made in the CDR3 loops in order to select for mutants for increased affinity to peptide MART-1/HLA-A2/Ig dimers. CDR3 libraries of the T1-S18 template were sorted with two variants of the MART-1 peptide: the nonamer spanning residues 27–35 (AAGIGILTV) and decamer spanning residues 26–35 which contained a modified anchor residue at position 2 (ELAGIGILTV). Some MART-1-specific TCRs have been shown to crossreact with nonamer, decamer, and anchor-modified decamer peptides[24–27]. After four rounds of selection by FACS with MART-1/HLA-A2/Ig dimers, various clones were isolated and examined for binding. Clone T1-S18.45, isolated with MART-1 (ELAGIGILTV)/HLA-A2, showed significant binding, whereas the template T1-S18 and wild-type T1 did not (Fig. 1B). Clone T1-S18.45 also showed the greatest binding improvement to the 9mer variant of the MART-1 peptide (AAGIGILTV) on the surface of yeast, despite isolation with the anchor-modified 10mer peptide (data not shown). Sequencing revealed that T1-S18.45 contained five mutations in CDR3α, from wild-type NDNAR to SSSDF (Supplementary Fig. 1).

Expression and binding studies of soluble T1 single-chain TCRs

The T1, T1-S18, and T1-S18.45 scTv genes (Vβ-linker-Vα) were cloned into an *E. coli* expression vector, induced to express the recombinant scTv, and refolded from inclusion bodies. Refolded preparations were purified by Ni-affinity and size exclusion chromatography, yielding scTv proteins of the expected monomeric molecular weight, 30 kDa (Fig. 2A). Surface plasmon resonance (SPR) was performed with immobilized scTv fragments to determine kinetics and binding affinities for the various MART-1 peptide variants. Kinetic titrations were performed in order to avoid regeneration steps. SPR data analysis revealed nanomolar affinities of T1-S18.45 for all three MART-1 peptide variants examined (Fig. 2B and 2C). The stabilized T1-S18 variant bound with micromolar affinities, yielding affinity improvements of the T1-S18.45 scTv of 700 to 4,500-fold for the ELAGIGILTV, ALGIGILTV, AAGIGILTV peptide complexes, respectively (Fig. 2C). The wild-type scTv T1 did not exhibit detectable binding, perhaps because it lacked adequate stability for immobilization and analysis. The micromolar affinities of the T1-S18, non-affinity matured TCR are in the same range as typically seen for most pep/MHC antigens.

To study whether the high-affinity, soluble scTv T1-S18.45 protein could detect peptide:HLA-A2 complexes on the surface of antigen presenting cells, the protein was biotinylated utilizing a biotin-succinimidyl cross-linking agent[28]. MART-1 (ELAGIGILTV) or null SL9 (SLYNTVATL) peptides were incubated with the TAP-deficient, HLA-$A2^+$ human cell line T2. Following peptide pulsing, T2 cells were stained with various concentrations of T1-S18.45- biotin followed by SA-PE, and analyzed by flow cytometry (Fig. 3). Specific staining for MART-1 was observed, yielding an estimated EC_{50} affinity measurement of 120 nM, similar to that observed by SPR.

Alanine scanning mutagenesis of Vα2-containing high affinity scTv fragments

The availability of three $V\alpha2^+$ TCRs with high-affinity (called A6, 868, and T1 here), each for a different peptide:HLA-A2 ligand, allowed us to compare the exact binding contribution of proposed key CDR1 and CDR2 residues among different TCRs. To examine the binding energetics of these peptide/MHC interactions, alanine mutants were generated at five positions in a panel of high affinity Vα2-containing scTv proteins: CDR1α residues D27, R28 and Q31, and CDR2α residues Y51 and S52. These residues have shown conservation

based on TCR sequence alignments[29] and the R28, Q31, Y51, and S52 side chains appear to be involved in contacts with the HLA-A2 helices in structures of three Vα2-containing TCRs (A6, Mel5, and DMF5; PDB files 1AO7, 3HG1, and 3QDG)(Fig. 4)[3–5]. Position D27 in CDR1α was the target of mutation for computationally-guided affinity increases in two Vα2-containing TCRs, A6 and DMF5[30; 31], and thus was also included in our analysis.

The yeast display system allows direct titrations of the fusion protein in order to examine affinities above a threshold K_D of about 1 μM, thereby avoiding the need to purify each protein or mutant[32]. Accordingly, each alanine mutant was titrated with various concentrations of cognate peptide:HLA-A2 and analyzed by flow cytometry (Fig. 5A,B,C). To compare mutants, the magnitude of changes in binding affinity were calculated from the concentrations of ligand that resulted in half-maximal binding of alanine mutant compared to 'wild-type' high-affinity scTv (Fig. 5D). For all three scTv fragments, the tyrosine at position 51 was the only residue that uniformly contributed significant binding (10 to ≥15-fold reductions in binding affinity of the alanine mutants) to the peptide:HLA-A2 interaction, regardless of the Vβ or the peptide. Other positions showed minimal effects for all three TCRs (R28A and S52A), an effect on only one TCR (D27A in the A6 TCR), or an effect on two TCRs (Q31A in the A6 and 868 TCRs). Thus, these positions showed plasticity in their contribution to the binding energy.

In order to determine if the effect of the Y51A mutation observed in the high affinity T1, A6, and 868 scTv fragments would hold true for the wild-type TCRs, variants of each scTv that contained the wild-type CDR loops, with or without the corresponding Y51A mutation, were cloned and expressed in *E. coli* (note that the affinities of wild-type scTv fragments are too low to detect binding in the yeast display format). The 868 scTv fragment and its Y51A mutant were unstable and could not be used to make reliable SPR measurements. However we were able to measure steady state equilibrium binding parameters for wild-type T1 and A6 single-chain TCRs and their Y51A mutants (stabilized versions of these scTv, without the affinity mutations). These binding measurements yielded a ΔΔG value of over −2 kcal/mol for Y51A of the T1 scTCR (i.e. K_D of 22 μM for the wild-type, whereas binding by the Y51A mutant could not be detected; hence, we estimated a K_D value of greater than 1 mM for the Y51A mutant). A ΔΔG value of - 0.8 kcal/mol for Y51A of the A6 scTCR was measured (K_D values of 2.2 μM and 10 μM for wild-type and Y51A, respectively). Additionally, a recent study by Piepenbrink and colleagues showed that the Y51A mutation in the wild-type full-length A6 TCR yielded a ΔΔG value of −0.6 kcal/mol mol, similar to that of the Vα2-containing DMF5 TCR[33]. Thus, we conclude that tyrosine 51 in the CDR2 of Vα2 contributes to binding energy among different TCRs in both the affinity-matured TCRs and the wild-type affinity TCRs.

Peptide:HLA-A2 selections from single-residue libraries of the MART-1 TCR

If during evolution the key CDR1 and CDR2 residues have been selected for MHC-binding, then peptide/MHC sorting of a yeast display library of all amino acids at these positions should yield enrichment for the evolutionary-driven residues. In order to determine the allowable residues at key positions of MHC restrictions, degenerate libraries (NNS codons) at Vα positions 31 and 51 positions were generated in the T1-S18.45 scTv yeast display vector. Each library underwent one round of selection with 45 nM MART-1/HLA-A2, and the top 8–10% binding clones were isolated by sorting. DNA from the enriched population of yeast was then subjected to 454 high-throughput sequencing to determine the amino acid frequency selected at each position (Fig. 5). At position 31, the wild-type glutamine was the highest frequency isolate, followed by alanine, cysteine, and valine (Fig. 5A). At position 51, tyrosine (wild-type), phenyalanine, and tryptophan were the highest frequency isolates (Fig. 5B). Thus, each positional library demonstrated a preference for the wild-type, energetically important residue, consistent with an evolutionary pressure to maintain these

residues. However, several other amino acids were also capable of supporting peptide/MHC binding.

Discussion

Crystal structures of different TCR:pep/MHC complexes have shown a conserved docking mode of TCR on the peptide/MHC such that the CDR1 and 2 loops are approximately positioned over the MHC helices and CDR3 over the peptide[1; 2; 29]. Evidence for the co-evolution of these molecules has been shown by the presence of conserved residues and interactions in specific positions in CDR1 and 2 loops[29]. In the current study we focused on the energetic contribution of putative conserved TCR residues that shape immune recognition.

During development in the thymus, T cells are required to bind to MHC within a narrow affinity range in order to pass both positive and negative selection. Mutagenesis studies have shown that single residue differences in CDR1 and 2 loops are able to shift T cells out of this affinity such that negative selection or death by neglect can occur[12; 29]. Although the TCR's intrinsic reactivity towards MHC could be partially masked by the negative selection process, structural and sequence data have suggested putative conserved residues that may play an important role in the binding energetics of the TCR:pep/MHC interface.

Higher affinity single-chain TCRs provide an approach to rapidly assess alanine mutants using yeast display and flow cytometry without the need to express large quantities of protein as has been done in previous studies[7–11; 34; 35]. Use of the higher affinity scTvs allows for detection of up to 100-fold decreases in affinity that would not otherwise be measurable within the normal range for TCR:pep/MHC (1–100 μM)[2]. Thus far, mutations that confer higher affinity on the variants of TCRs have most often been engineered in the CDR3 loops, in order to retain peptide specificity[19; 23; 36–41]. CDR1 loops often contact the MHC helices, and CDR2 loops tend to almost exclusively contact the MHC helices, and thus wild-type residues in these loops provide a good framework to access the binding energetics of "germline-encoded" contacts on HLA-A2 by alanine scanning mutagenesis. Furthermore, the structures of several high-affinity mouse TCRs in CDR3 residues have shown virtually identical CDR1 and CDR2 contacts with MHC as the wild-type TCR[16; 17]. Thus, we believe that the high-affinity TCRs provide a useful surrogate for these wild-type, germline encode residues in CDR1 and CDR2.

Our data showed that the putative conserved CDR2α residue Y51 contributed substantially to binding in all three of the high-affinity scTv fragments examined. Although tyrosine at this position is not abundant (in 12–16% of Vα regions), it interacts with the same region of MHC class I (near residue Q155) and class II (near βA73) in various structures[29]. Structural analysis of three Vα2-containing TCRs, A6[5], Mel5[4], and DMF5[3], suggest that the binding energy associated with mutation of Y51 to alanine could be due to the loss of contacts with HLA-A2α2 helix position(s) E154, Q155, and/or A158 (Fig. 4). In all three high-affinity scTv fragments examined, mutation to alanine led to a 10 to ≥15-fold decrease in binding affinity (≥1.4 kcal/mol in free energy).

While measurement of these interactions took advantage of the ability to use yeast display to rapidly analyze binding by the higher affinity variants, we were able to confirm the energetic role of the tyrosine at position 51 in wild-type scTv variants in which all CDR affinity mutations were reverted to the wild-type residues. These results are consistent with the similar docking and chemistries associated with wild-type TCR and affinity-matured TCRs in the 2C system, showing only the CDR3 loop with mutations in a different position [16; 17]. Although qualitatively we show that Y51 contributed to binding in both high-affinity and

wild-type TCRs, structural studies will be required to determine if there are changes in the docking of each of the loops and their residues. In this regard, a recent comparison of the A6 TCR/Tax/HLA-A2 complex with the high-affinity mutant A6-c134 TCR/Tax/HLA-A2 complex showed that the docking orientations were virtually identical[21]. While there were subtle changes in the positions the CDR3 and CDR2β loops, the positions of the CDR1α and CDR2α, and in particular the position of the side chain of Y51, were very similar (Supplemental Figure 2). In their report, the authors proposed that most of the increase in binding affinity of the A6-c134 TCR was achieved through a greater number of interactions between the CDR3 loops and the peptide.

As positive selection is associated with weak affinities and free energies in the range of ~4 kcal/mol[42], the contributions by this conserved tyrosine could reflect a substantial portion of this "minimal" affinity to ensure representation in the T cell repertoire. Early studies in the 2C system showed that affinities as low as 300 μM were able to yield agonist activity[43]. Additionally we have shown that a single peptide point substitution, F5R, in the peptide of the 2C TCR:QL9- L_d interaction reduced the affinity of the 2C TCR from 1.6 μM to 300 μM, yet the interaction was sufficient for agonist activity[44]. Based on this, it is reasonable to assume that an affinity of 1 mM (~4 kcal mol^{-1}) or even lower (higher K_D value) is likely to be in the range for positive selection.

Although binding analysis showed a consistent role of CDR2α residue Y51, more plasticity was observed in the contribution of CDR1α residues. For example, the D27A mutation led to a >15-fold decrease in binding of Tax/A2 by the A6 X15 TCR but this same mutation did not have a significant impact on binding by the other TCRs. The effect of the D27A mutation in the A6 TCR could be indirect, perhaps influencing the adjacent R28 through electrostatic interactions and affecting the stability of the entire loop. Similarily, the Q31A mutation caused a >15-fold change in both A6-X15 and T1-S18.45 TCRs, but the 868-Z11 TCR was not significantly affected. It has been proposed that CDR3 loops are able to modulate interactions of the MHC with germline derived CDR1 and 2 loops in a process termed 'CDR editing'[14]. This may account for the variation in binding contribution of residues in CDR1α. Analysis of contacts in crystal structure of Vα-containing TCRs DMF4, DMF5, A6, and Mel5 suggest a role of CDR1α in making peptide contacts in addition to MHC contacts[3; 4], which may also explain differences in the energetic contribution with different peptide ligands.

In order to further understand the requirements for binding, single codon yeast display libraries were generated at the two most energetically important CDR1 and CDR2 residues of the high-affinity T1-S18.45 TCR. The preferential selection of aromatic amino acids at position 51 (i.e. wild-type Tyr, Phe, and Trp) further supports the conservation at this position, and may reflect a general requirement for bulky amino acids which can adapt to varying MHC structures or chemistry[45]. However, position 31 showed a more diverse collection of allowable amino acids, from the chemical perspective, at this position (i.e. wild-type Gln, Cys, Ala, and Val). Nevertheless, the preferences for the wild-type residues in each case are consistent with their evolutionary pressures to retain these in the context of the Vα2:HLA-A2 interaction. This is not to say, however, that other CDR loops or residues would not suffice to provide the very low energy of interaction required for MHC-restriction through positive selection[13]. Clearly, the maintenance of the docking angle and general positions of each CDR loop in all wild-type TCRs and their high-affinity TCR counterparts suggests that the MHC interactions with CDR1 and CDR2 could collectively be capable of restraining the orientation of the complex, even in the absence of co-receptor (since the high-affinity TCRs described here were selected in the absence of CD8).

In a recent study comparing the crystal structures of G4 and E4 TCRs with their cognate ligand mutTPI-DR1, Mariuzza and coworkers showed that CDR2α made identical contacts with the MHC helices in both structures, but there was more plasticity or "wobble" in CDR1α loops, a finding that they attribute to the influence of the CDR3[14]. Our study provides a corresponding binding energy analysis that is consistent with these structural findings. Furthermore, we suggest that the energetically-conserved CDR2α residue (Y51) provides sufficient binding energy to contribute significantly to the very low affinity interactions required for positive selection. Although our study does not examine the importance of TCRβ chain residues or the impact of the co-receptor, crystal structures of the A6 TCR have suggested that the TCRα chain may dominate in the interaction with Tax/A2[5; 46–48]. The structures of the other two Vα2-positive TCRs described here are not known so it remains to be seen if their Vβ CDR1 and CDR2 play a more significant role than the A6 TCR.

Materials and methods

Antibodies, peptide:HLA-A2, and Flow Cytometry

Antibodies used to detect yeast surface expression included: anti-HA eptiope tag (Clone HA. 11; Covance), anti-Vβ16 antibody (Clone TAMAYA1.2; Beckman-Coulter), anti-Vα2 monoclonal antibody generated in our laboratory (data not shown), Goat-anti-mouse IgG $F(ab')_2$ AlexaFluor 647 secondary antibody (Invitrogen), and Streptavidin-phycoerythrin (SA:PE, BD Pharmingen). Peptides that bind to HLA-A2 [Tax_{11-19}: LLFGYPVYV, $SL9_{77-85}$ (HIV-Gag): SLYNTVATL, MART-$1_{26-35\ A27L}$: ELAGIGILTV, MART-1_{27-35}: AAGIGILTV27, and MART-$1_{27–35\ A28L}$: ALGIGILTV] were synthesized by standard F-moc (N-(9-fluorenyl)methoxycarbonyl) chemistry at the Macromolecular Core Facility at Penn State University College of Medicine (Hershey, PA, USA). For FACS and flow cytometry analysis, recombinant soluble dimeric HLA-A2:Ig fusion protein (BD DimerX) was used. Additionally, a monomeric HLA-A2-biotin reagent generated by the exchange of a UV-cleavable peptide for another HLA-A2-restricted peptide in the presence of UV light was utilized to determine fold changes of binding in alanine mutants on the surface of yeast cells[49; 50].

Cloning and expression of scTv in yeast display vectors

TCR variable region fragments (scTv) were expressed in yeast display plasmid pCT302 (Vβ-L-Vα)[51], which contains a galactose-inducable AGA2 fusion allowing for growth in Trp media. Induction of the scTv gene involves growth of the transformed EBY100 yeast cells to stationary phase in selection media followed by transfer to galactose-containing media. The T1 single-chain gene for TCRs was synthesized by Genscript (Piscataway, NJ, USA) with a F49S mutation in the Vα2-domain of the construct[19].

The MART-1-specific TCR genes were isolated from CTLs obtained by the MART- 1/HLA-A2 multimer-guided cloning as previously described[52]. The T1 scTv consisted of the variable contains attached by the linker region GSADDAKKDAAKKDGKS[19; 23; 53]. The scTv was introduced into the NheI and XhoI restrictions sites of pCT302 (Sequences in Supplementary Fig. 1).

Generation, display, and selection of mutated scTv yeast display libraries

Error-prone PCR was used to generate random mutations, as previously described[54]. CDR3 libraries were generated using Splicing by overlap extension (SOE) PCR spanning 5 adjacent codons at a time (2 libraries in each CDR3 loop)[55]. Pre-SOE PCR products were generated for each of the four libraries utilizing the following primer pairs. β1: 5'- GGC AGC CCC ATA AAC ACA CAG TAT -3' (Splice 4L) and 5'- TGA AGA GGC GCA AAA

ATA CAC ACC AGA ATC TTC CAG TTC GGC CGG TTG AAT TTT CAG GG -3', and 5'- GGA AGA TTC TGG TGT GTA TTT TTG CGC CTC TTC ANN SNN SNN SNN SNN SGT TGA ACA GTA TTT TGG TCC AGG TAC CCG TC -3' and 5'- TAA TAC GAC TCA CTA TAG GG -3' (T7); β2: Splice 4L and 5'- TGA AGA GGC GCA AAA ATA CAC ACC AGA ATC TTC CAG TTC GGC CGG TTG AAT TTT CAG GG -3', and 5'- GGA AGA TTC TGG TGT GTA TTT TTG CGC CTC TTC ACA TGC GGG TCT GNN SNN SNN SNN SNN STT TGG TCC AGG TAC CCG TCT GAC C -3' and T7; α1: Splice 4L and 5'- CAC CGC GCA CAG ATA AGT GGC TGA ATC AGA TGG TCG AGA ATC TCT AAT CAG CAG TGA AAC ATA CTG AGA -3', and 5'- CCA TCT GAT TCA GCC ACT TAT CTG TGC GCG GTG NNS NNS NNS NNS NNS CTG ATG TTT GGC GAT GGT ACC CAG CTG GTT GTG -3' and T7; α2: Splice 4L and 5'- CAC CGC GCA CAG ATA AGT GGC TGA ATC AGA TGG TCG AGA ATC TCT AAT CAG CAG TGA AAC ATA CTG AGA -3', and 5'- CCA TCT GAT TCA GCC ACT TAT CTG TGC GCG GTG AAT GAT NNS NNS NNS NNS NNS TTT GGC GAT GGT ACC CAG CTG GTT GTG -3' and T7. SOE PCR was performed with each corresponding Pre-SOE along with both T7 and Splice 4L for each library.

Yeast libraries were made by homologous recombination in EBY100 yeast by electroporating error prone or SOE PCR products along with NheI and XhoI digested pCT302[55]. The library was induced in galactose-containing media (SG-CAA) for 48 h, washed with 1 mL 1% PBS/BSA, and stained with the following: anti-HA eptiope tag (1:50), anti-Vα2 antibody (1:50), anti-Vβ16 antibody (1:50) along with goat-anti-mouse IgG F(ab')^2 AlexaFluor 647 secondary antibody (1:100), and corresponding peptide/HLA-A2 DimerX (100 nM) followed by SA-PE (1:100). Cells were washed (1 ml, 1% PBS/BSA), and the most fluorescent cells were selected using a FACS Aria (BD Bioscience) high-speed sorter. Selection was performed on the error prone library for anti-Vβ16 antibody staining (1:50). In order to test thermal stability of isolated clones, yeast were incubated at elevated temperature for 30 min prior to the staining protocol. Each individual CDR library was sorted for positive Vβ16-staining, pooled in equal cell numbers, and expanded. CDR3 library was selected with MART-1/HLA-A2 dimer (10–100 nM).

Expression in *E. coli*, refolding, and biotinylation of soluble scTv fragments

T1 wild-type, T1-S18, and T1-S18.45 were introduced into the pET28a expression vector with a N-terminal 6-His tag using NcoI and EcoRI restriction sites (forward primer: 5' TAT ACC ATG GGC AGC AGC CAT CAT CAT CAT CAT CAC AGC AGC GGC CTG GTG CCG CGC GGC AGC GAA GCT GGT GTT ACT CAA TTC 3', Reverse primer: 5' T TTA GAA TTC TTA AAT ATT CGG TTT CAC AAC CAG 3'). Plasmids were transformed into the BL21 cell line, expanded, and induced for expression. Following induction, cells were passed through a microfluidizer (Microfluidics Corporation, Newton, MA, USA), inclusion bodies were isolated, and protein was purified as previously described[56]. Soluble scTv were refolded and purified with Ni agarose resin (Qiagen, Valencia, CA) followed by gel filtration (Superdex 200). Folded scTvs were biotinylated using N-hydroxysuccinimide (NHS) biotin ester (EZ-Link Sulfo-NHS-LC-Biotin Kit, Pierce/Thermo Scientific). Biotinylation was verified by gel-shift with streptavidin by SDS– PAGE.

Binding of scTv proteins measured by surface plasmon resonance

The binding of purified refolded scTv proteins to cognate peptide/HLA-A2 was monitored with surface plasmon resonance (SPR) using a Biacore 3000 instrument. Peptide/HLA-A2 were generated by refolding from bacterially expressed heavy chain and β2m inclusion bodies as previously described[57]. Due to the high affinities of the scTv proteins, a kinetic titration assay was utilized, in which increasing concentrations of analyte were sequentially injected over the surface without the requirement for disruptive regeneration injections[58].

Experiments were performed with T1 wild-type (not detectable), T1-S18, and T1-S18.45-amine coupled to a standard CM5 sensor chip. Peptide/MHC analyte was sequentially injected at various concentrations. The amount of immobilized scTv was kept below 500 RU and the flow rate was set to the maximum of 100 ml/min to minimize mass transport effects. Data were analyzed using Biaevaluation 4.1 as described[58]. Solution conditions were 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P-20, 258C.

Binding of scTv fragments to peptide-pulsed antigen presenting cells

HLA-A2^{+} human cell line, T2 was incubated at 37 degrees for 2–3 hours with 1 μM MART-1 (ELAGIGILTV) or null SL9 (SLYNTVATL) peptide. Cells were then washed twice with 1% PBS/BSA, and incubated on ice for 1 hour with biotinylated scTv at various concentrations. Cells were washed twice with 1% PBS/BSA, followed by incubation with SA:PE for 30–45 min on ice. Cells were washed twice and analyzed using an Accuri C6 Flow Cytometer. Experiments were done with n=4.

Alanine scanning mutagenesis of scTvs

Four site-directed alanine mutants (CDR1α D27A, R28A and Q31A, and CDR2α Y51A and S52A) were introduced into previously described high affinity scTvs, A6 X15 and 868 Z11[19], and T1-S18.45 by site-directed mutagenesis using a Quikchange kit (Stratagene, La Jolla, CA). Yeast cells displaying the single-site mutants were titrated with cognate-peptide exchanged HLA-A2 monomers in triplicate at 8 nM, 40 nM, 200 nM, 1 μM, and 5 μM and analyzed by flow cytometry[49; 50]. Values were normalized using nonlinear regression analysis. Changes in binding affinity were approximated by determining the scTv concentrations at one-half maximal wild-type binding (5 μM). Independent experiments were performed with peptide/HLA-A2 dimers (BD DimerX) with similar results (data not shown).

Yeast display library generation, selection, and 454 sequencing of scTv libraries

Two libraries were generated in the high affinity T1-S18.45 scTv construct at CDR1α residue Q31 and CDR2α residue Y51 using SOE PCR as described above. Each library was stained with 45 nM MART-1 (ELAGIGITV)/HLA-A2 and sorted via FACS. Top staining (8–10%) clones were isolated, and amino acid sequences were determined by 454 sequencing (Roche/454 GS FLX+ Sequencer).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CDR	Complementarity determining region
HA	Hemagglutinin
HLA	Human leukocyte antigen

MHC	Major histocompatibility complex
scTv	Single-chain T cell variable fragment
scFv	Single-chain variable fragment
SPR	Surface plasmon resonance
T1	InRi-T1 T cell clone
TCR	T cell receptor
Vα/Vβ	Variable chain alpha / variable chain beta

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Highlights

- Several key residues of the TCR have been proposed to account for MHC-restriction
- Yeast display enabled engineering of high-affinity TCR against antigen MART-1
- Binding analysis of several TCRs revealed energetic importance of CDR2α residue Y51
- Plasticity in binding was observed for other CDR1α & CDR2α residues
- Evolutionary pressure for wild-type residues at key MART1 single-chain TCR residues
- Residue Y51 could contribute significantly to positive selection of all of the TCRs

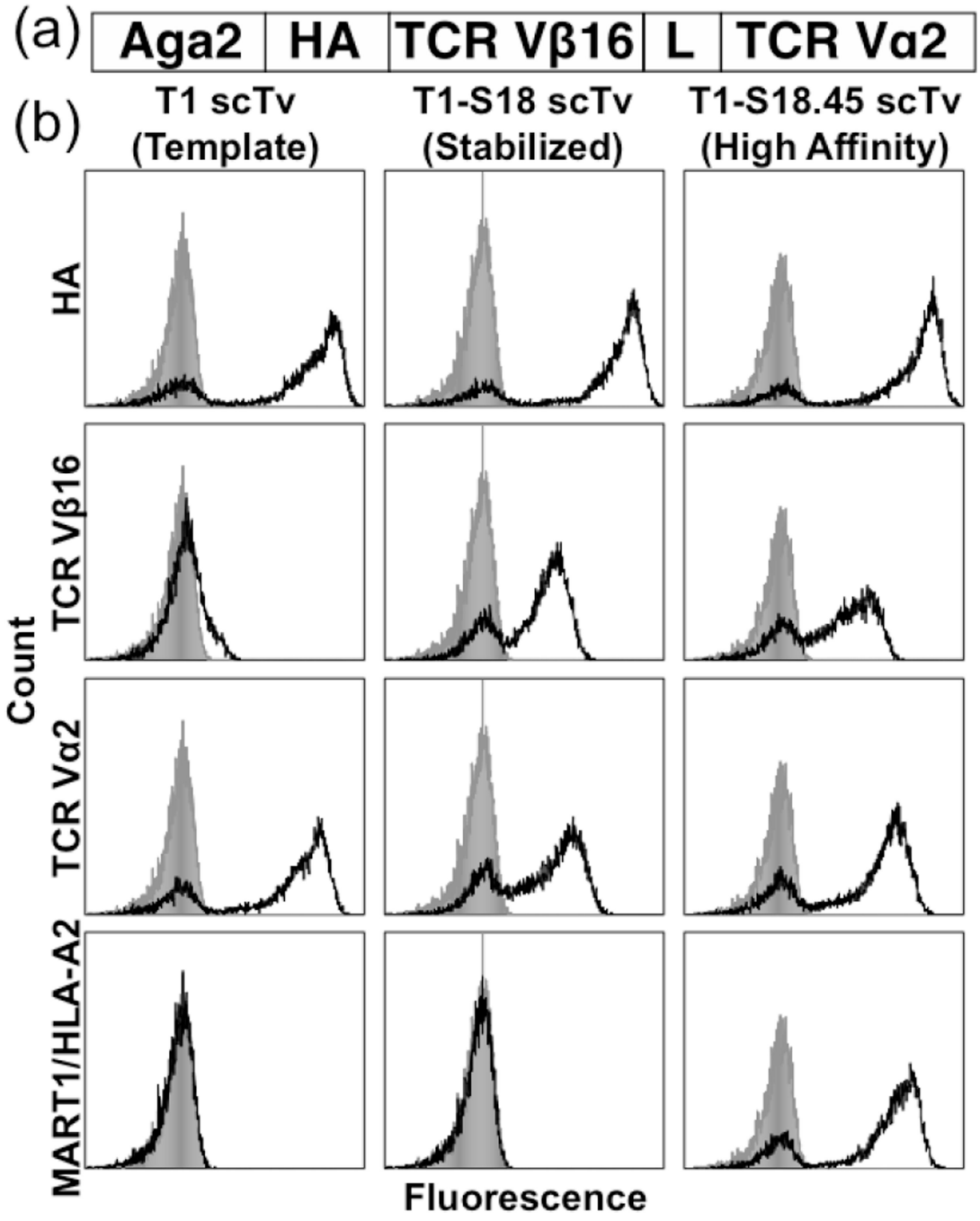
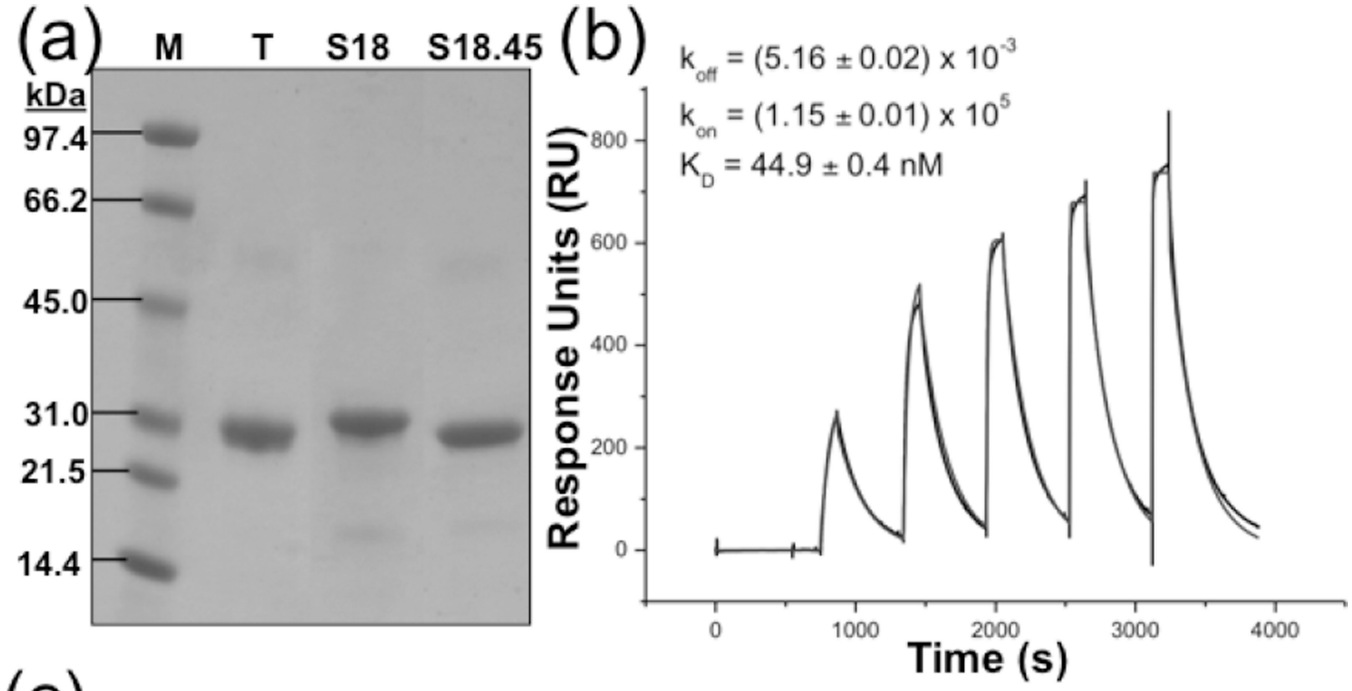


Figure 1. Yeast display and isolation of INR1-T1 (T1) single-chain TCR variable fragments (scTv)

(a) Schematic of scTv fusions for the human TCR, T1, which recognizes MART-1 peptides in presented in the context of HLA-A2. (b) The T1 TCR was cloned as a scTv and expressed on the surface of yeast (left panels). Yeast surface of scTV fusions were monitored for expression of an N-terminal tag [hemagluttanin (HA), black line] with anti-HA antibody and goat anti-mouse IgG alexa 647 secondary antibody or secondary only as a control (gray). The negative peak is is due to yeast that have lost plasmid, and serves as an internal control for each induced yeast sample. Cells were incubated with anti-Vβ16 and anti-Vα2 antibodies followed by goat anti-mouse IgG alexa 647 secondary antibody, or secondary only as a

control (gray). Cells were incubated with MART-1 peptide (ELAGIGILT)/HLA-A2 dimer at 100 nM. A random mutagenesis library was generated using the T1 scTv as a template and sorted with anti-Vβ16 antibody and goat anti-mouse IgG alexa 647 secondary antibody. Clone T1-S18 was isolated after two rounds of sorting (middle panels). Site directed libraries were made in CDR3 loop regions of the stabilized T1-S18 clone. The high affinity clone, T1-S18.45, was isolated following 4 rounds of sorting with MART-1 peptide (ELAGIGILT)/HLA-A2 dimer (right panels, black line). Histograms are representative of 3 or more experiments.



(c)

Peptide Variant	T1 wild type	T1-S18	T1-S18.45	Fold Change
ELAGIGILTV	Not detectable	32 μM	45 nM	710X
AAGIGILTV	Not detectable	210 μM	240 nM	880X
ALGIGILTV	Not detectable	≥1000 μM	220 nM	4500X

Figure 2. Purity and surface plasmon resonance of soluble MART-1-specific, single-chain TCR T1 and its engineered variants

T1 wt, T1-S18, and T1-S18.45 were expressed in the *E.coli* pET28 expression system, refolded from inclusion bodies, and purified by Ni-column and size exclusion chromatography. (a) SDS-PAGE of purified scTvs and molecular weight markers (M). (b) SPR trace of MART-1 (ELAGIGILTV)/HLA-A2 binding immobilized T1-S18.45. Fitted parameters (K_D, k_{on}, k_{off}) are shown in the inset. (c) Table showing the binding affinities of the T1-derived TCRs for variants of the MART-1 peptide.

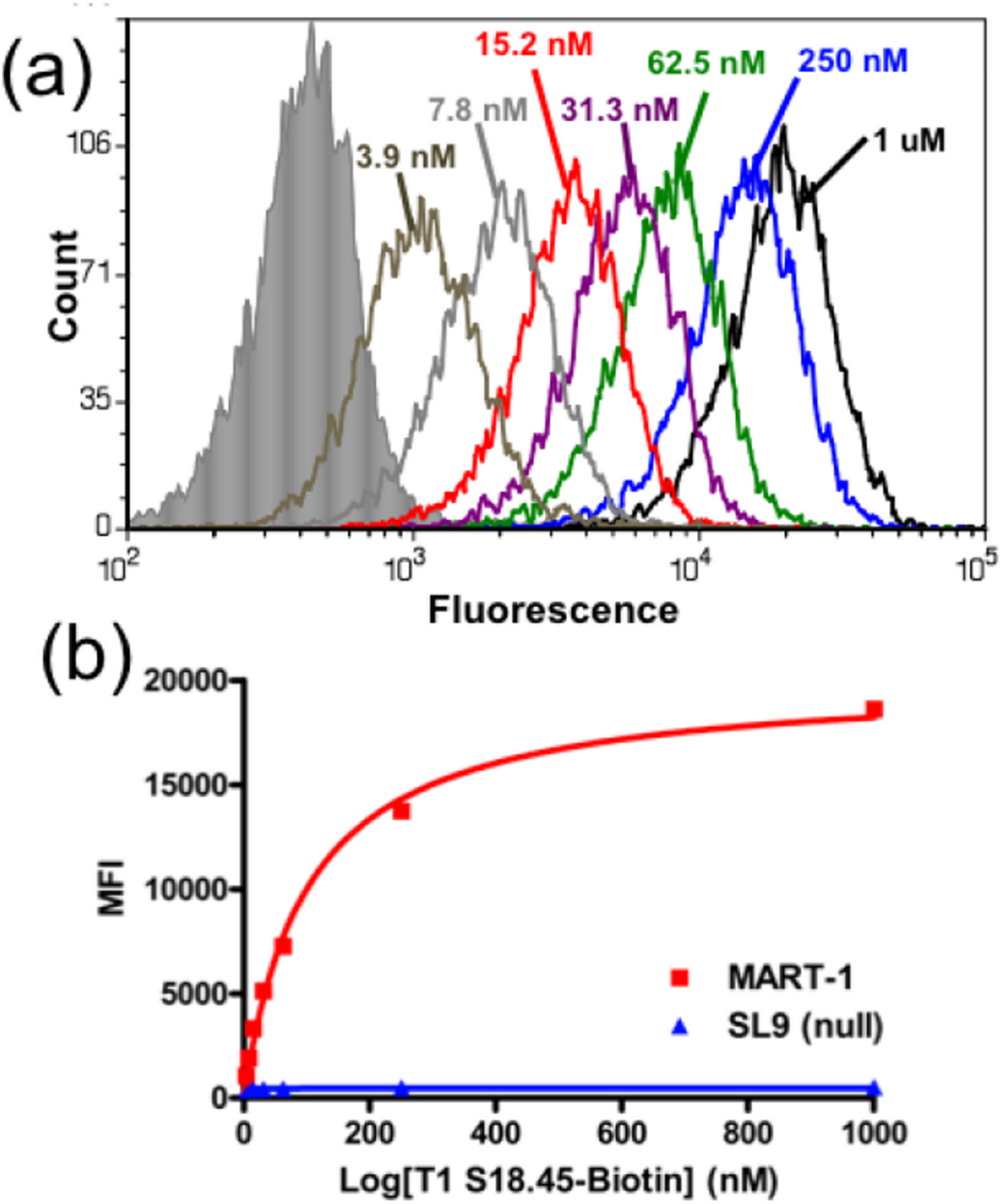


Figure 3. Binding of peptide-loaded antigen presenting cells by soluble T1-S18.45 scTv

(a) Titration of biotinylated T1-S18.45 scTv on antigen-presenting cell line T2 (HLA-A2+) preloaded with MART-1 peptide (1 μM) or null peptide, SL9 (1 μM). Cells were stained with 3.9 nM (tan), 7.8 nM (gray), 15.2 nM (red), 31.1 nM (purple), 62.5 nM (green), 250 nM (blue), or 1 μM (black) biotinylated T1-S18.45 scTv, followed by SA:PE. Data shown is representative of 4 experiments. (b) Mean fluorescence unit (MFU) values of histograms in (a) are plotted versus scTv-biotin concentration.

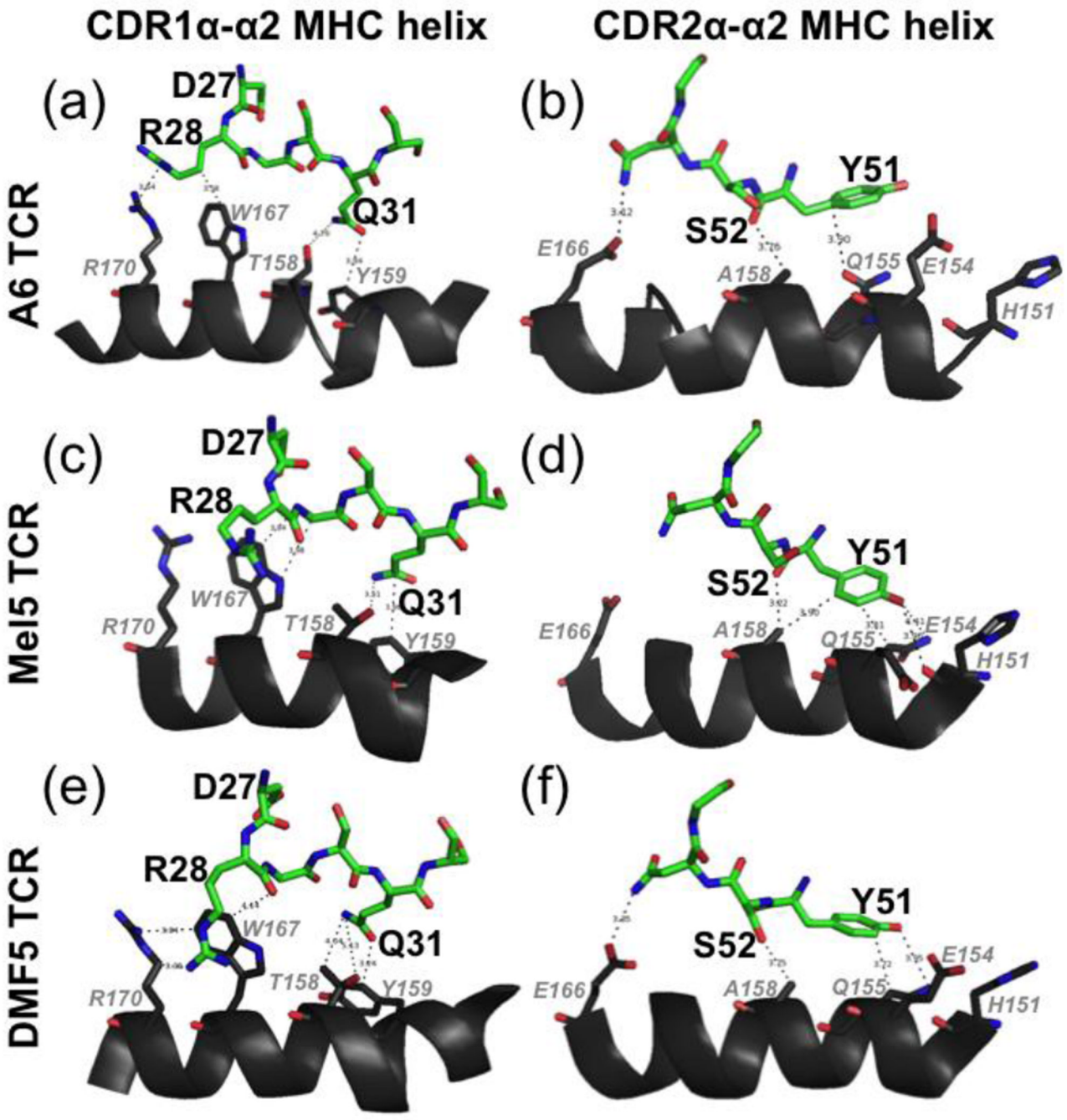


Figure 4. Crystal structures of Vα2-containing TCRs showing MHC contact positions in CDR1α and CDR2α loops

CDR1α residues D27, R28, and Q31 (a,c,e), and CDR2α residues Y51 and S52 (b,d,f) assessed in the alanine scanning study are highlighted in the structures of the A6 (a,b)[5], Mel5 (c,d)[4], and DMF5 (e,f)[3] TCRs, which all contain the Vα2 region. PDB files 1AO7, 3HG1, and 3QDG were used, respectively, in PyMol. TCR residue positions are indicated in black. MHC residues are italicized in gray.

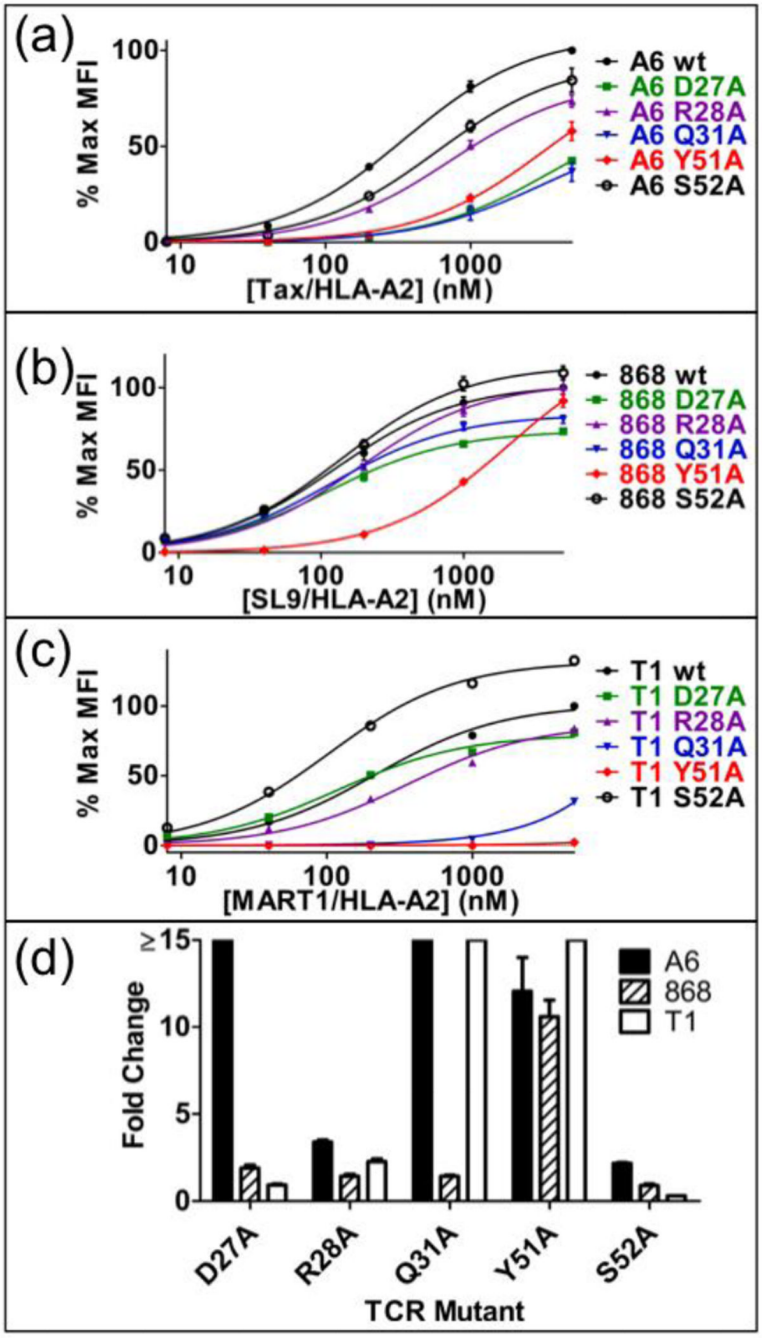


Figure 5. Alanine scanning mutagenesis of Vα2-containing single-chain TCRs

Binding titrations of yeast-displayed mutants of Vα2-containing single-chain TCR fragments (scTv): (a), A6-X15 specific for Tax/HLA-A2, (b) 868 Z11 specific for SL9/HLA-A2, and (c) T1-S18.45 specific for MART-1/HLA-A2. Alanine mutants were stained in triplicate with 8 nM, 40 nM, 200 nM, 1 μM, and 5 μM cognate peptide/HLA-A2 monomers followed by PE-conjugated streptavidin. Normalized percent max mean fluorescence intensity (MFI) is plotted against cognate peptide/HLA-A2 monomer concentration. Error bars represent standard deviations of triplicate experiments. (d) Fold changes in binding were determined by the scTv concentrations at one-half max wild-type

binding from titrations in Figure 4a,b,c. Error bars represent standard deviations of triplicate experiments.

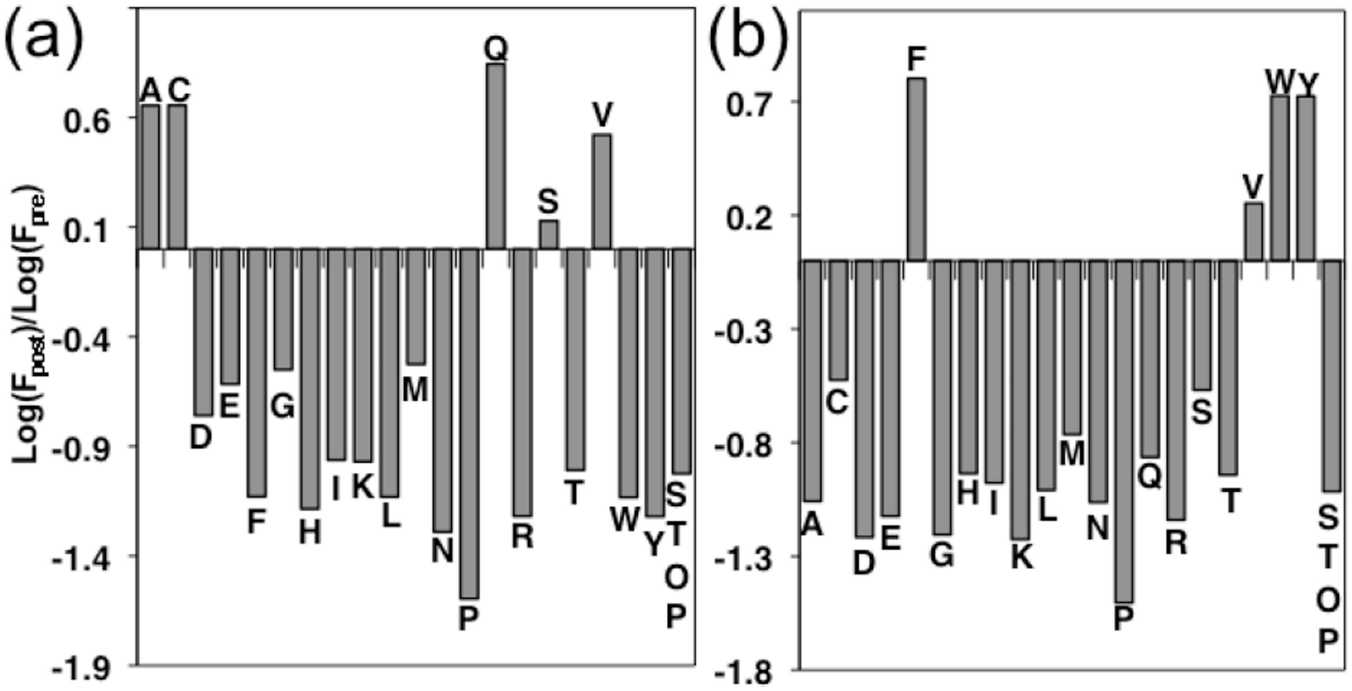


Figure 6. In vitro selected mutants of yeast-display libraries of the MART-1-specific TCR (T1-S18.45)

Yeast display libraries at position 31 in CDR1α (a) and position 51 in CDR2α (b) were generated, stained with 45 nM MART-1 (ELAGIGITV)/HLA-A2, and selected by fluorescence activated cell sorting. The top staining (8–10%) clones were isolated, and amino acid distribution was determined by 454 sequencing. Amino acid residues that were positively and negative selected are indicated on the y-axis as a function of the ratio of the logarithm of amino acid frequency post-selection divided by the logarithm of the frequency pre-selection. A total of 11,736 and 13,600 clones from the unselected position 31 library and selected position 31 library were sequenced, respectively; a total of 6,806, and 24,532 from the unselected position 51 library and selected position 51 library were sequenced, respectively.